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PATENTS ACT 1990

PROVISIONAL SPECIFICATION FOR THE INVENTION ENTITLED:

"MULTIPLEX SCREENING FOR LYSOSOMAL STORAGE DISORDERS (LSDs)"

This invention is described in the following statement:

BACKGROUND

The present invention is generally related to a multiple screening diagnostic for Lysosomal Storage Disorders ("LSDs") and related diseases. More particularly, this invention pertains to compounds, reagents, and methods for identifying and quantifying multiple target enzymes and proteins that are used to accurately diagnose LSDs. These target enzymes are naturally present in biological fluids or tissues of patients.

LSDs represent a group of over 40 distinct genetic diseases that generally affect young children. Individuals that are affected with a LSD present a wide range of clinical symptoms that depend upon the specific disorder or a particular genotype involved. The clinical symptoms associated with LSD's can have a devastating impact on both the child and the family of affected individuals. For example, central nervous system dysfunction, behavioral problems, and severe mental retardation are characteristic of many LSDs. Other clinical symptoms may include skeletal abnormalities, organomegaly, corneal clouding and dysmorphic features (Neufeld and Muenzer, 1995). Patients are usually born without the visible features of a LSD, but early stage symptoms can quickly develop into a progressive clinical concern. In severe cases, the affected children require constant medical management but still often die before adolescence.

The significance of LSDs to health care becomes obvious when comparing the group incidence rate for a LSD (1:5,000 births) to the group incidence rate of other with well-known and intensively studied genetic disorders, such as phenylketonuria (1:14,000) and cystic fibrosis (1:2,500), wherein these figures reflect incidence rates for Caucasian populations.

Once an individual begins to present the symptoms of a LSD, the actual clinical diagnosis of the disease is still a complex process. A clinical diagnosis of a LSD often requires multiple visits to a range of specialists, which can take months or even years. This long process is extremely stressful on the patient and family. Fortunately, there has been considerable progress in the diagnosis of LSDs over the past 20 years. For example, the development and introduction of chromatographic-based urine screens for a specific group of LSDs called mucopolysaccharidoses ("MPS") and oligosaccharidoses has facilitated screening of clinically selected patients for these disorders. Following a clinical index of suspicion for the disorders, the next stage of diagnosis involves a urine screen, wherein a "positive" urine screen is then followed by specific enzymatic analysis. Although the chromatographic-based screening methods are simple to perform, they

are relatively labor-intensive and often require experience to accurately interpret results. Consequently, chromatographic-based screening tests for LSDs are not used in some centers. Furthermore, these chromatographic-based screens are not readily amenable to automation, which has further limited their utilization in screening strategies for newborns.

The production of specific substrates and antibody capture assays has made the enzymatic analyses for LSDs more accurate. Although not wanting to be bound by theory, the majority of LSDs result from a reduction in levels of a particular enzyme(s) involved in a specific LSD, and the identification of the specific enzyme(s) deficiency will identify the specific form of LSD in the affected individual. The ability to quickly and accurately determine the levels of the more than 40 enzymes known to be involved with LSDs will assist in the development of better and more economical screening assays. Unfortunately, many of the chromatographic-based screens and enzyme assays mentioned above are time-consuming, invasive, complex, and require cultured cells, or tissue biopsies, which tends to make such assays inconvenient and expensive. As a result, testing for a LSD is often not a first line strategy for an affected child with early stage symptoms. Newborn screening for LSDs promises to provide early detection of the LSD, but all newborns must be screened in order to detect the disease early. Patients having a family history of LSDs may have a justifiable reason to perform an early screen for a LSD. However, the cost of an early screen of the LSD in individuals not having a family history may not be justified economically. Therefore, it is essential that any LSD screening process be feasible economically such that all newborns can be screened.

One common feature of LSDs is the accumulation and storage of materials within lysosomes. It is generally recognized that the accumulation and storage of material in LSD affected individuals results in an increase in the number and the size of lysosomes within a cell from approximately 1% to as much as 50% of total cellular volume. In non-affected individuals, such materials are typically degraded into degradation products within the lysosome and then transported across the lysosomal membrane. Certain lysosomal proteins are present at elevated levels in the lysosomes of affected individuals (Meikle *et al.*, 1997; Hua *et al.*, 1998). These identified proteins can be useful biomarkers for an early diagnosis of all LSDs. For example, sensitive immunoquantification assays have been developed to monitor the level of useful biomarkers such as the lysosome-associated membrane proteins ("LAMPs"), saposins, and α–glucosidase. Although the determination of either LAMP-1 or LAMP-2 levels alone in an 'at-increased-risk' group will identify up to 65% of LSD affected individuals, the combination of a LAMP with one

of the saposins increase identification of LSD affected individuals to approximately 85%. Therefore, a method to identify two or more biomarkers simultaneously would increase the accuracy of diagnosing a specific LSD as compared to any single assay. An automated multiplex assay that could perform a simultaneous screen on each of the known LSD deficient enzymes would reduce time and cost for accurate LSD diagnosis.

Multiplexing Bead Technology is built around 3 core technologies. The first is the family of fluorescently dyed microspheres having specific biomolecules bound to the surface of the microsphere. The second is a flow cytometer with 2 lasers and associated optics to measure biochemical reactions that occur on the surface of the microspheres, and the third is a high-speed digital signal processor to efficiently manage the fluorescent output. This type of system has been described in, for example: United States Patents 6,449,562; 6,524,793 and United States Patent Application SN 09/956,857. United States Patent 6,449,562 ("the '562 Patent") entitled "Multiplexed Analysis of Clinical Specimens Apparatus and Method," having Chandler *et al.* listed as inventors was issued on September 10, 2002. The '562 Patent discloses a method for the multiplexed diagnostic and genetic analysis of enzymes, DNA fragments, antibodies, and other biomolecules comprising the steps of constructing an appropriately labeled beadset, exposing the beadset to a clinical sample, and analyzing the combined sample/beadset by flow cytometry. Flow cytometric measurements are used to classify, in real-time, beads within an exposed beadset and textual explanations, based on the accumulated data obtained during real-time analysis, are generated for the user. The inventive technology of the '562 Patent enables the simultaneous, and automated, detection and interpretation of multiple biomolecules or DNA sequences in real-time while also reducing the cost of performing diagnostic and genetic assays. However, the '562 Patent does not describe how to utilize the technology for diagnosing a specific LSD.

United States Patent 6,524,793 ("the '793 Patent") entitled "Multiplexed Analysis of Clinical Specimens Apparatus and Method," having Chandler *et al.* listed as inventors, was issued on February 25, 2003. The '793 Patent discloses a method for the multiplexed diagnostic and genetic analysis of enzymes, DNA fragments, antibodies, and other biomolecules comprising the steps of constructing an appropriately labeled beadset, exposing the beadset to a clinical sample, and analyzing the combined sample/beadset by flow cytometry. Flow cytometric measurements are used to classify, in real-time, beads within an exposed beadset and textual explanations, based on the accumulated data obtained during real-time analysis, are generated for the user.

The '793 Patent enables the simultaneous, and automated, detection and interpretation of multiple biomolecules or DNA sequences in real-time while also reducing the cost of performing diagnostic and genetic assays. However, the '793 Patent does not describe how to utilize the technology for diagnosing a specific LSD.

United States Patent Application Serial No. 09/956,857 ("the '857 Application") entitled "Multiple Reporter Read-out for Bioassays" was published on March 20, 2003. The '857 Application describes a method for detecting a plurality of reactive sites on an analyte, comprising allowing reactants on an addressable microsphere and the reactive sites to react, forming reactant-reactive site pairs distinguishable by fluorescence intensity. The '857 Application also provides a method for detecting a plurality of analytes in a sample using addressable microspheres in combination with one or more reporter reagents. Also provided are a method for determining allele zygosity of a genetic locus having two alleles or more alleles using microparticles, and a method for detecting a plurality of SNPs in nucleic acid molecules. The '857 Application also provides a composition comprising an addressable microsphere carrying at least two fluorescent reactants capable of forming reactant-analyte pairs distinguishable by their fluorescence intensity, and kits comprising the inventive composition and a plurality of reporter reagents. However, the '857 Application does not describe how to utilize the technology for diagnosing a specific LSD. The entirety of each of the applications or patents listed above is hereby specifically incorporated by reference.

Accordingly, there is a need for the development of a fast, accurate and economical screen for early diagnosis of LSDs, which is amenable to automation. The ability to identify specific LSD enzymes in an automated multiplex assay will have a significant impact on the development of a newborn screening programs, as well as the ability to address a number of other issues associated with the early diagnosis and treatment of LSDs. The present invention provides compounds, reagents, and methods for a LSD diagnostic multiplex assay.

FIGURES

Figure 1A shows a microsphere having two spectrally distinct fluorophores, the target LSD capture antibody and the unique LSD target protein or target antigen bound to the target LSD capture antibody;

Figure 1B shows a microsphere capture sandwich immunoassay.

Figure 2 shows a calibration curve for α-glucosidase in a microsphere based assay;

Figure 3 shows multiplexed calibration curves in a microsphere based assay;

Figure 4A and 4B show calibration curves of α-glucosidase using bead technology and measured using Bio-Plex™ Protein Array system (Bio-Rad);

Figure 5 shows calibration curves for a tri-plex immune quantification of lysosomal proteins; and

Figure 6 shows target populations representing each LSD of interest analyzed.

SUMMARY

Lysosomal Storage Disorders ("LSDs") represent a group of over 40 distinct genetic diseases that generally affect young children. Individuals that are affected with a LSD present a wide range of clinical symptoms that depend upon the specific disorder or a particular genotype involved. The present invention is generally related to a multiple screening diagnostic for LSD and related diseases. More particularly, this invention pertains to compounds, reagents, and methods for identifying and quantifying multiple target enzymes and proteins that are used to accurately diagnose a LSD. These target enzymes and proteins are naturally present in biological fluids or tissues of patients. The invention also pertains to a Multiplexing Bead Technology for simultaneous screening of specific LSD enzymes.

A first aspect of the current invention is a composition used for diagnosing a LSD. The composition comprises a capture antibody capable of binding a target antigen, and a microsphere having the capture antibody conjugated to the microsphere. The target antigen is a LSD associated protein that comprises α-iduronidase, α-glucosidase, saposin C, LAMP-1, LAMP-2, β-glucosidase, α-galactosidase A, iduronate-2-sulphatase, N-acetylgalactosamine 4-sulphatase, galactose 6-sulphatase, acid sphingomyelinase, galactocerebrosidase, arylsulphatase A, saposin B, heparan-N-sulphatase, α-N-acetylglucosaminidase, acetylCoA: glucosamine N-acetyltransferase, N-acetylglucosamine 6-sulphatase, β-galactosidase, β-glucuronidase, aspartylglucosaminidase, acid lipase, β-hexosamindase A, β-hexosamindase B, GM2-acitvator, acid ceramidase, α-L-fucosidase, α-D-mannosidase, β-D-mannosidase, neuraminidase, phosphotransferase, phosphotransferase g-subunit, palmitoyl protein thioesterase, tripeptidyl peptidase I, cathespsin K, α-galactosidase B, or sialic acid transporter. The microsphere having

the conjugated capture antibody has a diameter of about 5 μm and at least a first fluorophore and a second fluorophore. The first fluorophore being spectrally distinct from the second fluorophore. The composition may further comprise a detection antibody, wherein the detection antibody is capable of binding the target antigen, but is different from the capture antibody, and the detection antibody is conjugated to a detectable label (e.g. a fluorescent label).

A second aspect of the current invention comprises a method for diagnosing a pre-clinical status, or a clinical status of a LSD. The method determines at least a first- and second- target antigen quantity from a target biological sample having an unknown clinical status of LSD. At least a first- and a second- reference antigen quantity are also determined from a reference biological sample having a known clinical status of LSD. The target antigens are LSD associated proteins that comprise α-iduronidase, α-glucosidase, saposin C, LAMP-1, LAMP-2, or other biomarkers associated with LSD. By calculating a target proportion between the first- and second- target antigen quantities, an adjusted target quantity can be assigned. Similarly, an adjusted reference quantity can be assigned by calculating a reference proportion between the first- and second- reference antigen quantities. The pre-clinical status or the clinical status of an LSD can then be determined by comparing a deviation of the adjusted target quantity to the adjusted reference quantity. In one specific embodiment, the target biological sample and the reference biological sample of this method are selected from a cellular extract, blood, plasma, or urine. Alternatively, the second target antigen and the second reference antigen comprise a biomarker indicator of cell number, organelle number, cell size, organelle size, cell volume, or organelle volume.

A third aspect of the current invention comprises a method for determining an amount of at least a first target antigen and at least a second target antigen indicative of a LSD in a target biological sample using a composition of capture antibody microspheres. The method comprises incubating at least a first capture antibody microsphere and at least a second capture antibody microsphere with the target biological sample forming a capture suspension. The first capture antibody microsphere and the second capture antibody microsphere are then recovered from the capture suspension. These first- and second- recovered microspheres are then hybridized with a first- and a second- detection antibody, respectively. The first recovered antibody microsphere and the second recovered antibody microsphere having a bound detection antibody can be detected when they are passed through an examination zone. Data is then collected that relates to one or more microsphere classification parameters, the presence or absence of the first- or second- detection antibody; and the amount of first- or second- detection antibody is quantified.

In a specific embodiment, the target biological sample is selected from a cellular extract, blood, plasma, or urine. In another specific embodiment, the first target antigen and second target antigens are each α-iduronidase, α-glucosidase, saposin C or other biomarkers associated with a LSD. The second target antigen may also comprise an indicator of cell number, organelle number, cell size, organelle size, cell volume, or organelle volume.

A fourth aspect of the current invention comprises a method of detecting multiple LSD target antigens in a sample. The specific subset of LSD antigens comprises α-iduronidase, α-glucosidase, saposin C or other biomarkers associated with LSD. The method comprises exposing a pooled population of target capture microspheres to the sample. Each of the target capture microspheres have distinct subsets, and each distinct subset has: (i) one or more characteristic classification parameters that distinguishes one target capture microsphere of one subset from those of another target capture microsphere subset according to a predetermined discriminate microsphere function table, which includes fluorescence emission intensities; and (ii) a distinct capture antibody that can bind a specific subset of LSD antigens. After the pooled population of target capture microspheres has been exposed to the sample, the exposed pooled population of target capture microspheres is passed through an examination zone. The identity and quantity of each specific subset of LSD target antigen of interest is determined, if present, in the sample by (i) collecting data relating to one or more subsets of target capture microsphere classification parameters that distinguishes one target capture antibody microsphere of one subset from those of another target capture antibody microsphere subset according to a predetermined discriminate function table, including the fluorescence emission intensities, (ii) collecting data relating to the presence or absence of a corresponding subset of specific LSD antigen, (iii) quantifying each corresponding subset of specific LSD antigen on each subset of capture antibody microsphere. In a specific embodiment, the method further comprises adding a pooled population of detection antibodies to the exposed pooled population of the target capture microspheres prior to passing the target capture microspheres through the examination zone.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Terms:

The term "a" or "an" as used herein in the specification may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

The term "animal," "subject," or "patient" as used herein may be used interchangeably and refers to any species of the animal kingdom. In preferred embodiments it refers more specifically to humans.

The term "clinical status" as used herein refers to patients that are being studied or treated by physicians for a LSD.

The term "comprise," or variations such as "comprises" or "comprising," as used herein may be used to imply the inclusion of a stated element or integer or group of elements or integers, but not the exclusion of any other element or integer or group of elements or integers.

The term "fluorophore" as used herein refers to any fluorescent compound or protein that can be used to quantify the LSD antigens.

The term "normalize" as used herein refers to bringing a target, reference, or other samples into conformity with a standard, pattern, model, etc. For example, in one embodiment, urine samples from LSD patients and non-LSD patients were normalized by using a 1 μmol equivalent of creatinine from each sample.

The term "phenotype" as used herein refers to the manifest characteristics of an organism collectively, including anatomical and psychological traits, that result from both its heredity and its environment.

The term "preclinical status" as used herein refers to the period of a disease before any of the clinical symptoms appear.

The term "lysosomal storage disorder ("LSD") associated protein" as used herein refers to any protein that has been linked to any LSD. In preferred embodiments, a LSD associated protein includes, but is not limited to α-iduronidase, α-glucosidase, saposin C, LAMP-1, LAMP-2, β-glucosidase, α-galactosidase A, iduronate-2-sulphatase, α-iduronidase, N-acetylgalactosamine 4-sulphatase, galactose 6-sulphatase, acid sphingomyelinase, galactocerebrosidase, arylsulphatase A, saposin B, heparan-N-sulphatase, α-N-acetylglucosaminidase, acetylCoA: glucosamine N-acetyltransferase, N-acetylglucosamine 6-sulphatase, β-galactosidase, β-glucuronidase, aspartylglucosaminidase, acid lipase, β-hexosamindase A, β-hexosamindase B, GM2-acitvator,

acid ceramidase, α-L-fucosidase, α-D-mannosidase, β-D-mannosidase, neuraminidase, phosphotransferase, phosphotransferase g-subunit, palmitoyl protein thioesterase, tripeptidyl peptidase I, cathespsin K, α-galactosidase B, or sialic acid transporter. As shown below, Table 1 indicates some enzyme deficiencies for LSDs.

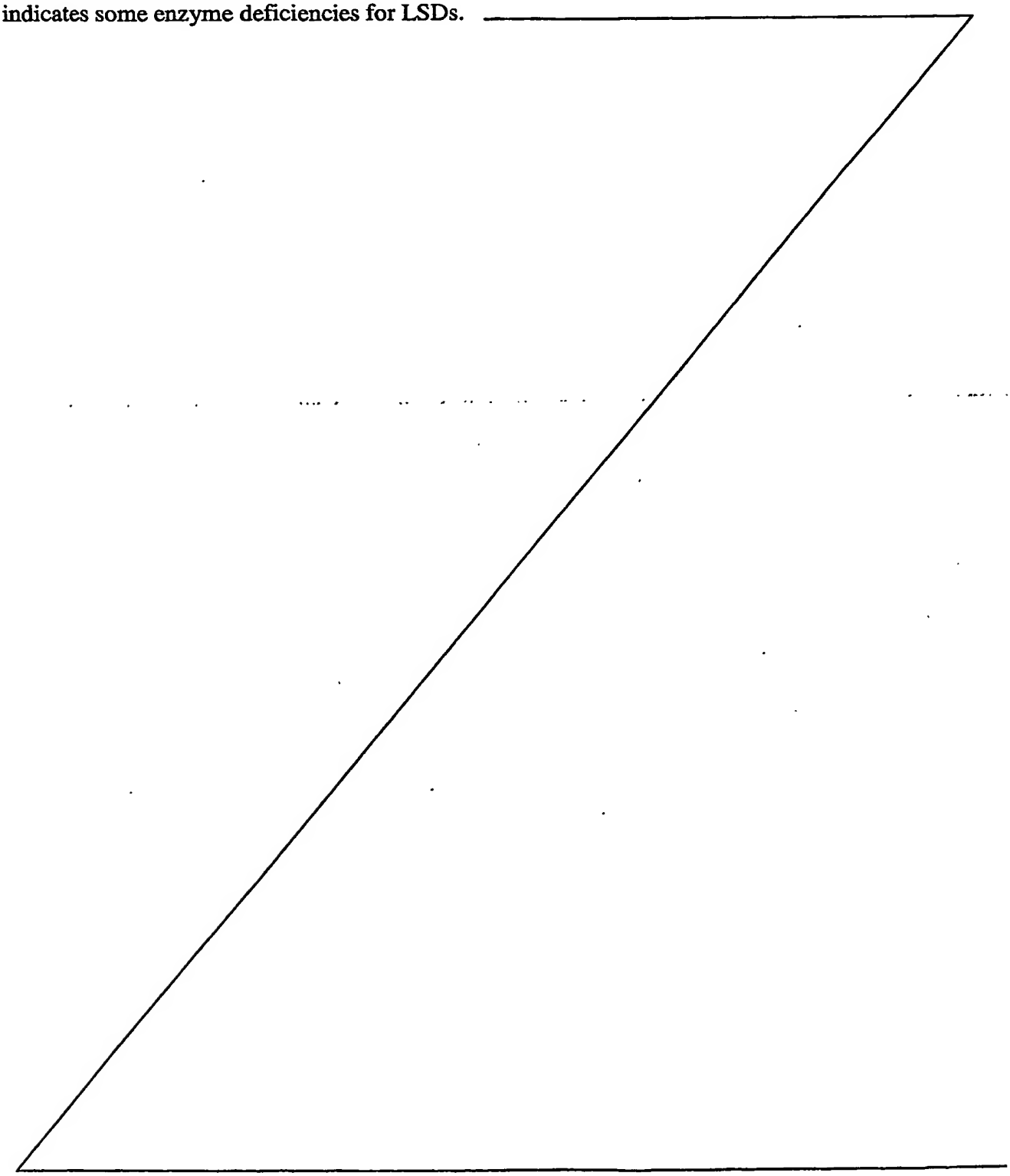


Table 1 Enzymes deficient in some common lysosomal storage disorders

Disease	Clinical Phenotype	Enzyme Deficiency	Australian Prevalence
Gaucher disease types I / II / III	Gaucher disease	Glucocerebrosidase (β-glucosidase)	1 in 57,000
Cystinosis		Cystine transporter	1 in 192,000
Fabry disease	Fabry disease	α-Galactosidase A	1 in 117,000
Glycogen storage disease II	Pompe disease	α-Glucosidase	1 in 146,000
Mucopolysaccharidosis type I	Hurler/Scheie syndrome	α-L-Iduronidase	1 in 88,000
Mucopolysaccharidosis type II	Hunter syndrome	Iduronate-2-sulphatase	1 in 136,000
Mucopolysaccharidosis type VI	Maroteaux-Lamy syndrome	N-acetylgalactosamine 4-sulphatase	1 in 235,000
Mucopolysaccharidosis type IVA	Morquio syndrome	Galactose 6-sulphatase	1 in 169,000
Niemann-Pick disease types A / B	Niemann-Pick disease	Acid sphingomyelinase	1 in 248,000
Globoid cell leucodystrophy	Krabbe disease	Galactocerebrosidase	1 in 201,000
Metachromatic leucodystrophy		Arylsulphatase A	1 in 92,000
Metachromatic leucodystrophy		Saposin B	
Mucopolysaccharidosis type IIIA	Sanfilippo syndrome	Heparan-N-sulphatase	1 in 114,000
Mucopolysaccharidosis type IIIB	Sanfilippo syndrome	α-N-Acetylglucosaminidase	1 in 211,000
Mucopolysaccharidosis type IIIC	Sanfilippo syndrome	AcetylCoA:N-acetyltransferase	1 in 1,407,000
Mucopolysaccharidosis type IIID	Sanfilippo syndrome	N-Acetylglucosamine 6-sulphatase	1 in 1,056,000
Mucopolysaccharidosis type IVB	Morquio syndrome	β-Galactosidase	
Mucopolysaccharidosis type VII	Sly	β-Glucuronidase	1 in 2,111,000
Niemann-Pick disease type C1	Niemann-Pick disease	Cholesterol trafficking	1 in 211,000
Niemann-Pick disease type C2	Niemann-Pick disease	Cholesterol trafficking	
Aspartylglucosaminuria		Aspartylglucosaminidase	1 in 2,111,000
Cholesterol ester storage disease	Wolman disease	Acid lipase	1 in 528,000
GM1-Gangliosidosis types I/II/III		β-Galactosidase	1 in 384,000
GM2-Gangliosidosis type I	Tay Sachs disease	β-Hexosaminidase A	1 in 201,000
GM2-Gangliosidosis type II	Sandhoff disease	β-Hexosaminidase A & B	1 in 384,000
GM2-Gangliosidosis		GM2-activator deficiency	
Farber Lipogranulomatosis	Farber disease	Acid ceramidase	
Fucosidosis		α-L-Fucosidase	> 1 in 2,000,000
Galactosialidosis types I / II		Protective protein	
α-Mannosidosis types I / II		α-D-Mannosidase	1 in 1,056,000
β-Mannosidosis		β-D-Mannosidase	
Mucolipidosis type I	Sialidosis types I / II	Neuraminidase	
Mucolipidosis types II / III	I-cell disease;	Phosphotransferase	1 in 325,000
Mucolipidosis type IIIC	pseudo-Hurler polydystrophy	Phosphotransferase g-subunit	
Mucolipidosis type IV		Unknown	
Multiple sulphatase deficiency		Multiple sulphatases	1 in 1,407,000
Neuronal Ceroid Lipofuscinosis, CLN1	Batten disease	Palmitoyl protein thioesterase	
Neuronal Ceroid Lipofuscinosis, CLN2	Batten disease	Tripeptidyl peptidase I	
Neuronal Ceroid Lipofuscinosis, CLN3	Vogt-Spielmeyer disease	Protein function not known	
Neuronal Ceroid Lipofuscinosis, CLN5	Batten disease	Protein function not known	
Neuronal Ceroid Lipofuscinosis, CLN8	Northern Epilepsy	Protein function not known	
Pycnodysostosis		Cathepsin K	
Sialic acid storage disease	Schindler disease	α-Galactosidase B	
Sialic acid storage disease	Sialuria; salla disease	Sialic acid transporter	1 in 528,000

Prevalence figures quoted from Miekle *et al.*, *JAMA* 281:249-254 (1999). Prevalence and ratio of lysosomal storage disorders may vary from country to country

The term "reference quantity" as used herein refers to a known, normalized amount of a MPS biomarker in a biological fluid. The reference quantity is determined from an animal, or group of animals having a defined clinical status, preclinical status, or phenotype of a MPS disease. The reference quantity may refer to a table compiled from various animals or groups of animals having correlations between relative amounts of MPS biomarkers in a biological fluid, and a known clinical status, preclinical status, or phenotype.

The LSDs represent a group of over 40 distinct genetic diseases that generally affect young children. Patients are usually born without the visible features of a LSD, but early stage symptoms can quickly develop into a progressive clinical concern. Although some effective LSD therapies have been developed, it is paramount that therapy be started as soon as the LSD has been diagnosed. Unfortunately, a clinical diagnosis of a LSD often requires multiple visits to a range of specialists requiring time-consuming, invasive, complex, inconvenient, and expensive assays. The current process for an accurate diagnosis of LSD for a patient not having a family history of LSD can take months to years, which is unacceptable when effective LSD therapies are needed earlier.

It is generally recognized that the accumulation of storage materials in the lysosomes of LSD affected individuals will increase from approximately 1% to as much as 50% of the total cellular volume. Certain lysosomal proteins are present at elevated levels in the affected individuals (Meikle *et al.,* 1997; Hua *et al.*, 1998). However, the current screening assays may be inaccurate because the variations in the numbers of lysosomes or white blood cell ("WBC") counts between individual samples are not typically considered. For example, an individual having a deficiency in a particular LSD biomolecule (e.g. lysosomal protein) but also having an unusually high WBC count or high numbers of lysosomes in the test sample may return an assay result that is consistent for individuals that do not have a LSD. Consequently, if WBC or high numbers of lysosomes were controlled in the sample preparation, a large inaccuracy could be avoided, and a proper diagnosis could be made.

Determining the quantities of multiple target enzymes increases the accuracy of diagnosing a specific LSD as compared to any single assay. For example, using immunoquantification assays directed toward identifying the levels of the lysosome-associated membrane proteins ("LAMPs"), such as LAMP-1 or LAMP-2, in an "at-increased-risk" group will identify up to 65% of LSD affected individuals. However, the combination of LAMPs with one of the

saposins increases identification of LSD affected individuals to approximately 85%. Therefore, a method to identify two or more biomarkers simultaneously would increase the accuracy of LSD diagnosis and reduce the time and cost for each assay.

EXAMPLE 1

Multiplexing Bead Technology and Target LSD Proteins. The Multiplexing Bead Technology is built around 3 core technologies. The first is the family of fluorescently dyed microspheres having bound biomolecules. The second is a flow cytometer with 2 lasers and associated optics to measure biochemical reactions that occur on the surface of the microspheres, and the third is a high-speed digital signal processor to efficiently manage the fluorescent output. Bio-Rad (Hercules, CA), provides a commercially available protein array system called the "Bio-Plex™". The Bio-Plex™ protein array system includes fluorescently dyed microspheres, a flow cytometer with 2 lasers and associated optics, and a high-speed digital signal processor. However, neither the Bio-Plex™ protein array system nor any other commercially available systems include any specific biomolecules, methods, compounds, or reagents needed for the simultaneous screening of specific LSD enzymes.

The Bio-Plex™ protein array system uses multiplexing technology to enable the simultaneous quantitation of up to 100 different analytes. This technology uses polystyrene microspheres internally dyed with differing ratios of 2 spectrally distinct fluorophores. Each fluorophore can have any of 10 possible levels of fluorescent intensity, thereby creating a family of 100 spectrally distinct bead sets. In a preferred embodiment, the dyed microspheres are conjugated with monoclonal antibodies specific for a target LSD protein or peptide thereof. Although not wanting to be bound by theory, each of the 100 spectrally distinct bead sets can contain a capture antibody specific for a unique LSD target protein. In a multiplexed Bio-Plex™ assay, LSD antibody-conjugated beads are allowed to react with the sample and a secondary LSD antibody, or a detection LSD antibody in a microtiter plate well to form a capture sandwich immunoassay. Figure 1A shows a drawing that represents the polystyrene microsphere (110) having the 2 spectrally distinct fluorophores, the target LSD capture antibody (120) bound to the microsphere, and the unique LSD target protein or target antigen (130) bound to the target LSD capture antibody. Figure 1B shows a drawing of the microsphere capture sandwich immunoassay, wherein a detection molecule (150) is bound to a detection LSD antibody (140), which is also bound to the target antigen (130), capture antibody (120) and the microsphere (110). The assay

solution is then drawn into the Bio-Plex™ array reader, which illuminates and reads the sample. Although not wanting to be bound by theory, there are many enzyme deficiencies specific for a particular LSD, and some of these enzymes are shown in Table 1. Specific capture antibodies (120), and detection antibodies (140) for the following target compounds are available: β-glucosidase; α-galactosidase A; iduronate-2-sulphatase; α-iduronidase; N-acetylgalactosamine 4-sulphatase; galactose 6-sulphatase; acid sphingomyelinase; galactocerebrosidase; arylsulphatase A; saposin B; heparan-N-sulphatase; α-N-acetylglucosaminidase; acetylCoA: glucosamine N-acetyltransferase; N-acetylglucosamine 6-sulphatase; β-galactosidase; β-glucuronidase; aspartylglucosaminidase; acid lipase; β-hexosamindase A; β-hexosamindase B; GM2-acitvator; acid ceramidase; α-L-fucosidase; α-D-mannosidase; β-D-mannosidase; neuraminidase; phosphotransferase; phosphotransferase g-subunit; palmitoyl protein thioesterase; tripeptidyl peptidase I; cathespsin K; α-galactosidase B; sialic acid transporter.

When a red diode "classification" laser (635 nm) in the Bio-Plex™ array reader illuminates a dyed bead, the bead's fluorescent signature identifies it as a member of one of the 100 possible sets. Bio-Plex™ Manager software correlates each bead set to the assay reagent that has been coupled to it (for example, a first LSD capture antibody coupled to bead set #22, and a second LSD capture antibody coupled to bead set #42). In this way the Bio-Plex™ protein array system can distinguish between the different assays combined within a single microtiter well. A green "reporter" laser (532 nm) in the array reader simultaneously excites a third fluorescent dye (phycoerythrin, "PE") bound to the detection LSD antibody in the assay. Although not wanting to be bound by theory, the amount of green fluorescence is proportional to the amount of target analyte captured in the immunoassay. Extrapolating the captured amount of target analyte to a standard curve allows quantitation of each LSD analyte in the sample. The digital signal processing algorithms provide simultaneous real-time data acquisition of classification and reporter signal output from thousands of beads per second, supporting up to 100 x 96 = 9,600 analyte measurements from each 96-well plate.

EXAMPLE 2

Specific target capture microspheres and target reporter antibodies. The BioPlex Protein Array System was used to demonstrate the type and nature of the reagents necessary for a LSD multiplex diagnostic assay. Three target proteins (e.g. LAMP-1, saposin C, and α-glucosidase) were used to design target capture microspheres and target reporter antibodies.

The monoclonal capture antibody for LAMP-1 was BB6 provided by Sven Carlsson (Carlsson *et al.*, 1989). The monoclonal reporter antibody for α-glucosidase (43D1) was obtained from Pharming, Inc. and has been described (Fransen *et al.*, 1988).

The polyclonal reporter antibody for LAMP-1, the rabbit polyclonal reporter antibody for saposin C, the sheep polyclonal capture antibody for α-glucosidase, and the monoclonal capture antibody ("7B2") for saposin C were prepared within the Lysosomal Diseases Research Unit at the WCH in Adelaide, Australia using standard techniques, known in the art, and briefly described below.

POLYCLONAL ANTIBODIES. Sheep polyclonal antibody was produced against recombinant proteins. A sheep was injected sub-cutaneously with 2mg of protein in 1 mL of an emulsion of phosphate buffered saline (pH 7.4) and complete Freunds adjuvant, followed by four booster injections (2mg each) with incomplete Freunds adjuvant, each three weeks apart. One week after the last injection the sheep was bled out and serum collected. Rabbit polyclonal antibody was produced in the same manner, except 0.2-1.0 mg of protein was used per immunisation.

Sheep polyclonal antibody was purified on a 5 mL Hitrap ™ Protein G affinity column (Pharmacia Biotech, Uppsala, Sweden) followed by an affinity column prepared from the recombinant protein used for the immunisation. Recombinant protein affinity columns were prepared by coupling 5 mg of the recombinant protein to 2.5 mL of Affi-gel 10 (Bio-Rad, Hercules, CA, USA) as per manufacturer's instructions.

Briefly, 5 mL of sheep serum was diluted with 5 mL of phosphate buffered saline (pH 7.4) and centrifuged (2200g, 10 min, 4°C). The centrifuged serum was passed through a 0.2 μm filter, and then loaded on to the Protein G column at a flow rate of 0.5 mL/min. The column was washed with phosphate buffered saline, pH 7.4 and the antibody eluted with 0.1 mol/L H_3PO_4/NaH_2PO_4, pH 2.5 and immediately neutralised by adding 1.0 mol/L Na_2HPO_4 ($1/10^{th}$ vol). The protein content was estimated by absorbance at 280nm (absorbance = 1.4 for 1.0 g/L of protein). The eluate was diluted four fold and then loaded on to the appropriate recombinant protein affinity column at the same flow rate. The column was washed and eluted as described for the Protein G column.

MONOCLONAL ANTIBODIES. Monoclonal antibodies were produced in Balb/C mice using standard immunisation protocols (Harlow *et al.*, 1988). Mice were immunised with recombinant enzyme using established protocols. Plasma cells from these immunised mice were fused with P3.653 myeloma cells (Zola *et al.*, 1982) and the resulting hybridoma cell lines screened for antibodies against the recombinant protein by direct ELISA (Harlow *et al.*, 1988).

Monoclonal antibodies were purified from cell culture supernatants by ammonium sulfate precipitation followed by affinity purification on Hitrap™ Protein G affinity column (Pharmacia Biotech, Uppsala, Sweden).

Coupling capture antibodies to specific microsphere beads. The target capture antibodies were coupled to Bio-Rad carboxylated ("COOH") beads as follows: anti-LAMP-1 to bead #(17), anti-saposin C to bead #(19), and anti-α-glucosidase to bead #(21). The coupling of the target capture antibodies to the polystyrene microspheres was performed using the BioRad bead coupling kit (Catalog number 171-406001, BioRad, Hercules, CA). The Bio-Plex™ amine coupling kit includes 4 ml bead wash buffer, 85 ml bead activation buffer, 135 ml PBS, pH 7.4, 10 ml blocking buffer, 25 ml storage buffer, 105 ml staining buffer, 40 coupling reaction tubes. The Bio-Plex™ amine coupling kit provides the buffers necessary to covalently couple 6–150 kD proteins to 5.5 μm dyed carboxylated polystyrene beads in under 5 hr. The covalent couple of the target capture antibody to the carboxylated polystyrene bead is achieved via carbodiimide reactions involving the protein primary amino groups and the carboxyl functional groups bound on the surface of polystyrene beads. The covalent attachment is permanent, leaving no unbound protein after cleanup, even after months of storage. The protein-coupled beads can then be used in multiplex protein-protein binding studies or in the development of multiplex assays that can be analyzed with the Bio-Plex™ protein array system. The bead yield per coupling reaction is approximately 80%, or enough protein-coupled beads for two 96-well microtiter plates using 5,000 beads per well.

Once the coupling reaction was completed, the target capture antibody-coupled beads were enumerated and the efficiency of the protein coupling reaction was validated, according to the manufacturer's protocol with modifications. In this procedure, the protein-coupled beads were reacted with a phycoerythrin ("PE")-labeled antibody that binds to the coupled protein, which was then analyzed using the Bio-Plex™ protein array system. This procedure was performed by reacting the beads with a PE-labeled antibody. Alternatively, a reaction using a biotinylated

antibody followed by streptavidin-PE may be used. Although not wanting to be bound by theory, the intensity of the fluorescent signal of this reaction is directly proportional to the amount of protein on the surface of the beads. A successful coupling typically yields a mean fluorescent intensity ("MFI") signal that is greater than 2,000. The protein coupling validation procedure provided a rapid relative assessment of the amount of protein coupled to the beads, but could not verify the functionality of the protein.

Coupling of the phycoerythrin reporter molecule to the detection antibodies in the LAMP-1, saposin C and α-glucosidase assays was achieved using the Molecular Probes (Eugene, Oregon, USA) Protein-Protein Coupling Kit, as per manufacturer's instructions with modifications. There are several published methods known in the art for preparation of phycobiliprotein conjugates with antibodies and other proteins. Generally, the coupling chemistry used to crosslink a phycobiliprotein to another protein includes: (a) treating the antibody or other protein with a succinimidyl ester maleimide derivative at pH 7.5, which converts some lysine residues of the antibody to thiol-reactive maleimides; (b) preparing a thiolated phycobiliprotein by reducing the appropriate SPDP-modified phycobiliprotein with dithiothreitol ("DTT") or with tris-(2-carboxyethyl)phosphine ("TCEP"); (c) mixing the above two dialyzed protein conjugates to yield a stable thioether crosslink; and (d) chromatographically separating the phycobiliprotein conjugates from the unreacted proteins.

Development of multiplex assays. A calibration curve was generated using liquid calibrator proteins in a microsphere based assay using calibrator protein capture antibodies and bead sets #17, #19 and #21 respectively (BioRad, Hercules, CA, USA). Figure 2 shows a calibration curve for a single assay for α-glucosidase. The detection capability for the amount of calibrator protein present in each well reaction was linear in the range of 0 to 4 ng/well of the assay. The MFI was the average of the total fluorescence detected for all beads in the defined bead region. Calibration curves were also established, using liquid calibrators, for LAMP-1 (open square), saposin C (open circle), and α-glucosidase (open triangle), as shown in Figure 3. Increased MFI for the α-glucosidase protein, when compared to Figure 2, is the result of improvements in the capture antibody labeling of the microspheres and the phycoerythrin reporter labeled antibodies.

Figure 3 also indicates that the detection capability for a multiplex assay of three calibrators was linear from 0 to 2 ng/well of the assay. The sensitivity of the microsphere assay system was

also demonstrated with the target capture sheep polyclonal antibody for α-glucosidase and bead set (#19) using a biotinylated reporter antibody with streptavidin-phycoerythrin conjugate (Molecular Probes #S-866). As shown in Figure 4, α-glucosidase was detectable down to a level of 10 pg /well using this assay. Figure 4A shows the calibration curve in the range 0-2.5 ng/well, and Figure 4B shows the same calibration curve expanded in the range 0-0.156 ng/well.

EXAMPLE 3

Tri-plex Assay for the Determination of α-Iduronidase, α-Glucosidase and Saposin C. A high sensitivity, tri-plex assay for target antigens α-iduronidase, α-glucosidase, and saposin C was developed using the microsphere technology based upon Luminex LABMAP™ technology.

Specific Target Capture Microspheres and Target Reporter Antibodies. Specific target capture microspheres and target reporter antibodies were produced using antibodies directed against three specific target proteins (e.g. α-iduronidase, α-glucosidase, and saposin C). The sheep anti-α-iduronidase and anti-α-glucosidase polyclonal antibodies were initially purified by ammonium sulphate precipitation. The ammonium sulphate precipitation purified antibodies were further purified using a protein G affinity purification (Amersham Pharmacia 5ml #17-0404-01). The protein G affinity purified antibodies were finally purified using an Hi trap NHS-activated HP column (Amersham Pharmacia 5ml #17-0717-01) coupled with either a α-iduronidase or α-glucosidase protein. The monoclonal antibodies anti-α-iduronidase, anti-α-glucosidase, and anti-saposin C were purified from hybridoma supernatant using protein G affinity purification according to manufacturer's specifications (Amersham Pharmacia 5ml #17-0404-01).

Sheep anti-α-iduronidase and, anti-α-glucosidase polyclonal antibodies and anti-saposin C monoclonal antibody (7B2) were coupled to dyed polystyrene beads using the antibody protein amino group via carbodiimide chemistry according to manufacturer's instructions at a concentration of 9μg of IgG to 1.4×10^6 beads. There are several published methods known in the art for efficiently biotinylating antibodies and other proteins. However, the purified anti-α-iduronidase (Id1A), anti-α-glucosidase (43D1), and anti-saposin C (S13C1) monoclonal antibodies were biotinylated using manufacturer's instructions for a FluoReporter® Biotin-XX Protein labeling kit F-2610 purchased from Molecular Probes (Eugene, OR). Generally, the

FluoReporter® Biotin-XX Protein Labeling Kit contains a biotin-XX succinimidyl ester, which reacts with primary amines of proteins or other biomolecules to form stable biotin conjugates. The long spacer between the biotin and the re-active group in biotin-XX succinimidyl ester enhances the ability of the conjugated biotin to interact with the relatively deep biotin-binding sites of avidin and streptavidin. The biotinylated protein was purified from the excess biotin using a gel filtration column. The degree of biotinylation was determined using an avidin–HABA complex and a control biotinylated goat IgG.

Development of Triplex Assays. LSD target antigen capture microspheres were diluted in PBS containing 1% BSA (assay buffer). The diluted LSD target antigen capture microspheres were then added to stock beads in a 96 well filtration plate (Millipore #MABVS1210), wherein the diluted LSD target antigen capture microspheres and stock beads had a total volume of 1µl per well. Each microwell containing the beads was then washed 3 times with PBS containing 0.05% Tween 20 (wash buffer) under vacuum using a manifold (Millipore #MAVM096OR). Standard solutions containing α-iduronidase, α-glucosidase, and saposin C protein (50µl) were added in serial 2-fold dilutions in assay buffer, as indicated. Biotinylated antibodies (50µl) were added to each well, wherein the final concentration of each antibody was 16ng/well in assay buffer. The plate was covered and incubated for 2 hours at room temperature with shaking. The wells were washed, incubated with Streptavidin R-phycoerythrin conjugate (Molecular Probes # S-866) (50ng/well) in assay buffer for 10 minutes at room temperature with shaking. After a final wash, 125µl of assay buffer was added per well and the plate shaken for 5 minutes at room temperature. Fluorescence was measured using the Bio-Plex™ Protein Array system in combination with the Bio-Plex™ software version 2.0 (Bio-Rad, Hercules, CA). Figure 5 shows the resulting calibration curves for α-iduronidase (open circle), α-glucosidase (open square), and saposin C (open triangle) of the tri-plex assay.

EXAMPLE 4

Multiplex Method to Screen the Newborn Population for Major LSDs. The following is an example of a single Multiplex assay suitable for use in North America and Europe. Twelve specific LSDs were chosen because of their relatively high prevalence in North America and Europe, together with the availability of effective therapies that would benefit from early diagnosis. The Multiplex could, for example, test for the following 14 target proteins, wherein the associated LSD is shown in parentheses: LAMP-1 (generic LSD), saposin C (generic LSD),

α-glucosidase (Pompe), α-galactosidase A (Fabry), glucocerebrosidase or β-glucosidase (Gaucher), α-iduronidase (MPS I), iduronate-2-sulphatase (MPS II), heparan-N-sulphatase (MPS IIIA), α-N-acetylglucosaminidase (MPS IIIB), galactose-6-sulphatase (MPS IVA), β-galactosidase or galactocerebrosidase (Krabbe), galactose-3-sulphatase (MLD), sphingomyelinase (Niemann-Pick A/B) and N-acetylgalactosamine-4-sulphatase (MPS VI).

The multiplex technology will enable the combination of LSD screened for to be changed as treatment methods improve, new LSD are identified or needs in different geographic areas change.

The person skilled in the art would appreciate that antibodies to all of the proposed 14 proteins would be required for the Multiplex assay.

The present invention improves the accuracy and detection of all of these LSDs and can for example be completed in a single multiplex assay using the Multiplex assay. LAMP-1 and saposin C are used as markers to normalize the population for the lysosomal content of the patient sample. For some disorders, these proteins may provide additional discriminatory power by showing an increase in concentration relative to the non-disease state. By calculating the ratio of these proteins to the individual proteins deficient in each LSD, greater discriminatory power can be attained. This concept can be extended beyond the calculation of ratios of individual proteins to the determination of protein profiles which look at all protein concentrations determined for a given sample. The use of discriminate analysis or other statistical methods can provide improved discrimination between control and affected populations.

It is to be understood that proteins characteristic of other LSD types can replace, or add to, the 14 lysosomal proteins listed above and that such modifications may depend on the frequency of individual LSDs for particular geographic regions. For example, the relative prevalence of individual LSDs is different in North America, Japan and China.

It is also to be understood that other biomolecules, such as DNA or RNA, or protein activities may be used or measured for the purposes of this invention.

EXAMPLE 5

In one embodiment of the invention a series of lysosomal proteins (e.g. LAMP-1, saposin C, α-glucosidase, β-glucosidase, α-galactosidase, α-iduronidase, iduronate-2-sulphatase and N-acetylgalactosamine-4-sulphatase) are multiplexed. Samples from a control population ($n \geq 100$) are analyzed with the multiplexed assay to determine the normal range for each of the analytes. Samples from a target population (Pompe disease affected individuals) ($n \geq 20$) are also analysed to determine the reference range of each analyte in this population. The level of each analyte in the target population is identified as being elevated, decreased or unchanged, relative to the control population. This provides a protein profile or fingerprint for the disease state (Pompe disease). Target populations representing each LSD of interest are analysed and profiles/fingerprints obtained, as shown in Figure 6. Unknown samples are then analysed and the resulting patterns compared with the available profiles to identify the specific LSD.

EXAMPLE 6

In one embodiment of the invention a series of lysosomal proteins (LAMP-1, saposin C, α-glucosidase, β-glucosidase, α-galactosidase, α-iduronidase, iduronate-2-sulphatase and N-acetylgalactosamine-4-sulphatase) are multiplexed. Samples from a control population ($n \geq 100$) are analysed with the multiplexed assay to determine the normal range for each of the analytes. Each analyte is normalised to the general lysosomal markers (LAMP-1 and saposin C) in addition to the other specific markers to produce a series of ratios. These ratios are then used to provide a profile of the control population. Samples from a target population (Gaucher disease affected individuals) ($n \geq 20$) are also analysed and the results normalised as described. The specific ratios that best differentiate the control and target populations are then utilised develop a profile/fingerprint of the disease state (Gaucher disease). A similar process is performed for each LSD of interest. Samples from individuals with unknown LSD status are analysed and the profile compared with each disease profile to identify the individual as either unaffected by an LSD of affected by a specific LSD.

EXAMPLE 7

In one embodiment of the invention a series of lysosomal proteins (LAMP-1, saposin C, α-glucosidase, β-glucosidase, α-galactosidase, α-iduronidase, iduronate-2-sulphatase and N-

acetylgalactosamine-4-sulphatase) are multiplexed. Samples from a control population ($n \geq 100$) are analysed with the multiplexed assay to determine the normal range for each of the analytes. Samples from a target population (Gaucher disease affected individuals) ($n \geq 20$) are also analysed to determine the reference range of each analyte in this population. The two sets of data are used as a training set to perform discriminate analysis and identify a discriminate function that will enable the separation of the disease affected individuals from the control population. The discriminate function will rely on some or all of the analytes in the profile. Using the same process, discriminate functions for each LSD of interest are developed. Analyte profiles from individuals with unknown LSD status will be evaluated with the discriminate function to determine the likelihood of being affected by a specific LSD.

EXAMPLE 8

In one embodiment of the invention a series of lysosomal proteins (LAMP-1, saposin C, α-glucosidase, β-glucosidase, α-galactosidase, α-iduronidase, iduronate-2-sulphatase and N-acetylgalactosamine-4-sulphatase) are multiplexed. Samples (e.g., dried blood spots) from all newborns in a population are analysed and assigned a probability of being affected by a specific LSD based on protein profiles/fingerprints of discriminate functions as described in Examples 5,6 and 7.

EXAMPLE 9

In one embodiment of the invention a series of lysosomal proteins (LAMP-1, saposin C, α-glucosidase, β-glucosidase, α-galactosidase, α-iduronidase, iduronate-2-sulphatase and N-acetylgalactosamine-4-sulphatase) are multiplexed. Samples from a control population ($n \geq 100$) are analysed with the multiplexed assay to determine the normal range for each of the analytes. Samples from a target population (Gaucher disease affected individuals) ($n \geq 20$) are also analysed to determine the reference range of each analyte in this population. The two sets of data are used as a training set to perform discriminate analysis and identify a discriminate function that will enable the separation of the Gaucher disease affected individuals from the control population. The discriminate function for each Gaucher patient is correlated to the disease severity (phenotype) to provide a prediction of phenotype (disease progression) in asymptomatic patients.

In a second embodiment, samples taken from an affected individual at different times during the course of therapy are analysed. The discriminate function is used to determine the degree of normalisation of the protein profile for that individual (how close does it approach the control profile) and thereby monitor the efficacy of therapy.

EXAMPLE 10

In one embodiment of the invention a series of lysosomal proteins (LAMP-1, saposin C, α-glucosidase, β-glucosidase, α-galactosidase, α-iduronidase, iduronate-2-sulphatase and N-acetylgalactosamine-4-sulphatase) are multiplexed. Samples from a control population ($n \geq 100$) are analysed with the multiplexed assay to determine the normal range for each of the analytes. Samples from a target population (cancer affected individuals) ($n \geq 20$) are also analysed to determine the reference range of each analyte in this population. The two sets of data are used as a training set to perform discriminate analysis and identify a discriminate function that will enable the separation of the affected individuals from the control population. The discriminate function is then used to identify unknown patients having a protein profile consistent with the particular cancer under investigation. This embodiment thereby provides early identification of the cancer.

In another embodiment, the target group represent individuals known to be at high risk for the development of a specific cancer and the discriminate function is used to identify individuals with increased susceptibility for the development of the specific cancer.

As shown in the above examples of the multiplex concept combined with the protein profile/fingerprint concept, there are many ways the profile can be analysed. Levels of proteins, ratios of proteins and discriminate analysis have been described, but other examples could include the use of neural networks. Therefore, it will be readily apparent to one skilled in the art that various substitutions and modifications may be made in the invention disclosed herein without departing from the scope and spirit of the invention. This invention may also be applied to a number of other conditions while still using lysosomal proteins as markers.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A composition used for diagnosing a lysosomal storage disorder ("LSD") comprising:

a capture antibody capable of binding a target antigen; and

a microsphere having the capture antibody conjugated to the microsphere;

wherein,

the target antigen comprises an LSD associated protein; and

the microsphere comprises at least a first fluorophore and a second fluorophore.

2. The composition of claim 1, further comprising a detection antibody, wherein the detection antibody is capable of binding the target antigen, but is different from the capture antibody; and the detection antibody is conjugated to a fluorescent label.

3. The composition of claim 1, wherein the target antigen is α-iduronidase, α-glucosidase, saposin C, LAMP-1, or LAMP-2.

4. The composition of claim 1, wherein the target antigen is β-glucosidase, α-galactosidase A, iduronate-2-sulphatase, N-acetylgalactosamine 4-sulphatase, galactose 6-sulphatase, acid sphingomyelinase, galactocerebrosidase, arylsulphatase A, saposin B, heparan-N-sulphatase, α-N-acetylglucosaminidase, acetylCoA: glucosamine N-acetyltransferase, N-acetylglucosamine 6-sulphatase, β-galactosidase, β-glucuronidase, aspartylglucosaminidase, acid lipase, β-hexosamindase A, β-hexosamindase B, GM2-acitvator, acid ceramidase, α-L-fucosidase, α-D-mannosidase, β-D-mannosidase, neuraminidase, phosphotransferase, phosphotransferase g-subunit, palmitoyl protein thioesterase, tripeptidyl peptidase I, cathespsin K, α-galactosidase B, or sialic acid transporter.

5. The composition of claim 1, wherein the first fluorophore is spectrally distinct from the second fluorophore.

6. The composition of claim 1, wherein the microsphere has a diameter of about 5 μm.

7. A method for diagnosing a pre-clinical status, or a clinical status of a lysosomal storage disorder ("LSD") in a target animal comprising:

(a) determining at least a first target antigen quantity from a target biological sample of the target animal;

(b) determining at least a second target antigen quantity from the target biological sample of the target animal;

(c) assigning an adjusted target quantity by calculating a target proportion between the first target antigen quantity and the second target antigen quantity;

(d) obtaining a first reference antigen quantity of a reference biological sample from a reference animal, or group of reference animals, having a known LSD pre-clinical or clinical status;

(e) obtaining a second reference antigen quantity of a reference biological sample from a reference animal, or group of reference animals, having a known LSD pre-clinical or clinical status;

(f) assigning an adjusted reference quantity by calculating a reference proportion between the first reference antigen quantity and the second reference antigen quantity;

(g) comparing a deviation of the adjusted target quantity to the adjusted reference quantity;

wherein,

the first target antigen is the same or equivalent to the first reference antigen;

the second target antigen is the same or equivalent to the second reference antigen; and

the deviation of the adjusted target quantity from the adjusted reference quantity is a pre-

clinical or clinical indication of a specific LSD.

8. The method of claim 7, further comprising repeating steps a-g for n numbers of rounds, wherein,

n is an integer greater than 1;

each consecutive round of repeated steps replaces a word "first" to a term that reflects a (2 + r) number of rounds, and r is an integer that represents the number of rounds completed; and

each consecutive round of repeated steps replaces a word "second" to another term that reflect a (3 + r) number of rounds, and r is an integer that represents the number of rounds completed.

9. The method of claim 7, wherein the target biological sample and the reference biological sample is selected from a cellular extract, blood, plasma, or urine.

10. The method of claim 7, wherein the first target antigen and the first reference antigen are each α-iduronidase, α-glucosidase, saposin C, LAMP-1, or LAMP-2.

11. The method of claim 7, wherein the second target antigen and the second reference antigen are each α-iduronidase, α-glucosidase, saposin C, LAMP-1, or LAMP-2.

12. The method of claim 7, wherein the first target antigen and the first reference antigen are each β-glucosidase, α-galactosidase A, iduronate-2-sulphatase, α-iduronidase, N-acetylgalactosamine 4-sulphatase, galactose 6-sulphatase, acid sphingomyelinase, galactocerebrosidase, arylsulphatase A, saposin B, heparan-N-sulphatase, α-N-acetylglucosaminidase, acetylCoA: glucosamine N-acetyltransferase, N-acetylglucosamine 6-sulphatase, β-galactodidase, β-glucuronidase, aspartylglucosaminidase, acid lipase, β-hexosamindase A, β-hexosamindase B, GM2-acitvator, acid ceramidase, α-L-fucosidase, α-D-

mannosidase, β-D-mannosidase, neuraminidase, phosphotransferase, phosphotransferase g-subunit, palmitoyl protein thioesterase, tripeptidyl peptidase I, cathespsin K, α-galactosidase B, or sialic acid transporter.

13. The method of claim 7, wherein the second target antigen and the second reference antigen are each β-glucosidase, α-galactosidase A, duronate-2-sulphatase, α-iduronidase, N-acetylgalactosamine 4-sulphatase, galactose 6-sulphatase, acid sphingomyelinase, galactocerebrosidase, arylsulphatase A, saposin B, heparan-N-sulphatase, α-N-acetylglucosaminidase, acetylCoA: glucosamine N-acetyltransferase, N-acetylglucosamine 6-sulphatase, β-galactosidase, β-glucuronidase, aspartylglucosaminidase, acid lipase, β-hexosamindase A, β-hexosamindase B, GM2-acitvator, acid ceramidase, α-L-fucosidase, α-D-mannosidase, β-D-mannosidase, neuraminidase, phosphotransferase, phosphotransferase g-subunit, palmitoyl protein thioesterase, tripeptidyl peptidase I, cathespsin K, α-galactosidase B, or sialic acid transporter.

14. The method of claim 7, wherein the second target antigen and the second reference antigen are each an indicator of cell number, organelle number, cell size, organelle size, cell volume, or organelle volume.

15. The method of claim 7, wherein the second target antigen and the second reference antigen each comprise LAMP-1, LAMP-2, saposin C, CD45 leukocyte common antigen, LIMP II, CD63.

16. A method for determining an amount of at least a first target antigen and at least a second target antigen indicative of lysosomal storage disorder ("LSD") in a target biological sample, the

method comprising:

(a) incubating at least a first capture antibody microsphere and at least first a second capture antibody microsphere with the target biological sample forming a capture suspension;

(b) recovering the first capture antibody microsphere and the second capture antibody microsphere from the capture suspension forming a first recovered microsphere and a second recovered microsphere;

(c) hybridizing the first recovered microsphere and the second recovered microsphere with at least first a first detection antibody and at least first a second detection antibody, respectively, forming a detection suspension;

(d) recovering the first recovered antibody microsphere and the second recovered antibody microsphere from the detection suspension forming a first detected microsphere and a second detected microsphere;

(e) passing the first detected microsphere and second detected microsphere through an examination zone;

(f) determining a first quantity of the first detection antibody associated with the first detected microsphere, and a second quantity of the second detection antibody associated with the second detected microsphere by (i) collecting data relating to one or more microsphere classification parameters, (ii) collecting data relating to the presence or absence of the first- or second- detection antibody on the first- or second- recovered microsphere; and (iii) quantifying the amount of first- or second- detection antibody on the first- or second- recovered microsphere;

wherein,

the first capture antibody microsphere comprises a first capture antibody conjugated to a first microsphere, and the second capture antibody microsphere comprises a second capture antibody conjugated to a second microsphere;

the first microsphere is spectrally distinct from the second microsphere;

the first capture antibody and the first detection antibody are distinct, but each binds a first LSD associated target antigen;

the second capture antibody and the second detection antibody are distinct, but each binds a second LSD associated target antigen;

the first target antigen and second target antigen are different;

the first detection antibody is conjugated to a first fluorescent detection label;

the second detection antibody is conjugated to the first fluorescent detection label or a second fluorescent detection label;

the quantity of the first- or second- detection antibody is proportional to the amount the first- or second- target antigen in the target biological sample.

17. The method of claim 16, wherein the target biological sample is selected from a cellular extract, blood, plasma, or urine.

18. The method of claim 16, wherein the first target antigen is α-iduronidase, α-glucosidase, or saposin C.

19. The method of claim 16, wherein the second target antigen is α-iduronidase, α-glucosidase, or saposin C.

20. The method of claim 16, wherein the first target antigen is β-glucosidase, α-galactosidase A, Iduronate-2-sulphatase, α-iduronidase, N-acetylgalactosamine 4-sulphatase, galactose 6-sulphatase, acid sphingomyelinase, galactocerebrosidase, arylsulphatase A, saposin B, heparan-N-sulphatase, α-N-acetylglucosaminidase, acetylCoA: glucosamineN-acetyltransferase, N-

acetylglucosamine 6-sulphatase, β-galactosidase, β-glucuronidase, aspartylglucosaminidase, acid lipase, β-hexosamindase A, β-hexosamindase B, GM2-acitvator, acid ceramidase, α-L-fucosidase, α-D-mannosidase, β-D-mannosidase, neuraminidase, phosphotransferase, phosphotransferase g-subunit, palmitoyl protein thioesterase, tripeptidyl peptidase I, cathespsin K, α-galactosidase B, or sialic acid transporter.

21. The method of claim 16, wherein the second target antigen is β-glucosidase, α-galactosidase A, uiduronate-2-sulphatase, α-iduronidase, N-acetylgalactosamine 4-sulphatase, galactose 6-sulphatase, acid sphingomyelinase, galactocerebrosidase, arylsulphatase A, saposin B, heparan-N-sulphatase, α-N-acetylglucosaminidase, acetylCoA: glucosamine N-acetyltransferase, N-acetylglucosamine 6-sulphatase, β-galactosidase, β-glucuronidase, aspartylglucosaminidase, acid lipase, β-hexosamindase A, β-hexosamindase B, GM2-acitvator, acid ceramidase, α-L-fucosidase, α-D-mannosidase, β-D-mannosidase, neuraminidase, phosphotransferase, phosphotransferase g-subunit, palmitoyl protein thioesterase, tripeptidyl peptidase I, cathespsin K, α-galactosidase B, or sialic acid transporter.

22. The method of claim 16, wherein the second target antigen is an indicator of cell number, organelle number, cell size, organelle size, cell volume, or organelle volume.

23. The method of claim 16, wherein the second target antigen comprise LAMP-1, LAMP-2, saposin C, CD45 leukocyte common antigen, LIMP II, CD63.

24. The method of claim 16, wherein the microsphere has a diameter of about 5um.

25. A method of screening for lysosomal storage disorder ("LSD") in a target biological sample comprising:

(a) determining a target quantity of a target biomolecule from the target biological sample of a target animal;

(b) determining a cell quantity of a cell marker from the target biological sample of a target animal;

(c) assigning an adjusted target quantity to the target biomolecule by calculating a target proportion between the target quantity of the target biomolecule and the cell quantity of the cell marker;

(d) obtaining a reference quantity of a reference biomolecule from the reference biological sample of a reference animal;

(e) obtaining a cell quantity of a cell marker from the reference biological sample of a reference animal;

(f) assigning an adjusted reference quantity to the reference biomolecule by calculating a reference proportion between the reference quantity of the reference biomolecule and the cell quantity of the cell marker;

(g) comparing the adjusted target quantity to an adjusted reference quantity;

wherein,

the target biomolecule comprises a LSD associated protein; and

the target biomolecule is the same or equivalent to a reference biomolecule;

the cell marker is the same or equivalent to a cell marker reference;

a deviation of the adjusted target quantity from the adjusted reference quantity is a pre-clinical or clinical indication of a specific LSD.

the target quantity of the target biomolecule comprises an amount or activity level of the target biomolecule.

the cell quantity of the target biomolecule comprises an amount or activity level of the cell marker;

the cell marker being an indicator of cell number, organelle number, cell size, organelle size, cell volume, or organelle volume.

26. The method of claim 25, wherein the target biological sample is selected from a cellular extract, blood, plasma, or urine.

27. The method of claim 25, wherein the target biomolecule is α-iduronidase, α-glucosidase, or saposin C.

28. The method of claim 25, wherein the target biomolecule is β-glucosidase, α-galactosidase A, Iduronate-2-sulphatase, α-iduronidase, N-acetylgalactosamine 4-sulphatase, galactose 6-sulphatase, acid sphingomyelinase, galactocerebrosidase, arylsulphatase A, saposin B, heparan-N-sulphatase, α-N-acetylglucosaminidase, acetylCoA: glucosamine N-acetyltransferase, N-acetylglucosamine 6-sulphatase, β-galactosidase, β-glucuronidase, aspartylglucosaminidase, acid lipase, β-hexosamindase A, β-hexosamindase B, GM2-acitvator, acid ceramidase, α-L-fucosidase, α-D-mannosidase, β-D-mannosidase, neuraminidase, phosphotransferase, phosphotransferase g-subunit, palmitoyl protein thioesterase, tripeptidyl peptidase I, cathespsin K, α-galactosidase B, or sialic acid transporter.

29. The method of claim 25, wherein the cell marker comprises a protein indicator of cell number, organelle number, cell size, organelle size, cell volume, or organelle volume.

30. The method of claim 25, wherein the cell marker comprises: LAMP-1, LAMP-2, saposin

C, CD45 leukocyte common antigen, LIMP II, CD63.

31. The method of claim 25, wherein the target biomolecule or cell marker comprises an intracellular biomolecule.

32. A method of detecting multiple Lysosomal Storage Disease ("LSD") target antigens in a sample, the method comprising:

(a) exposing a pooled population of target capture microspheres to the sample, the target capture microspheres having distinct subsets, and each distinct subset having: (i) one or more characteristic classification parameters that distinguishes one target capture microsphere of one subset from those of another target capture microsphere subset according to a predetermined discriminate microsphere function table, which includes fluorescence emission intensities; and (ii) a distinct capture antibody that can bind a specific subset of LSD antigens;

(b) passing the exposed pooled population of target capture microspheres having distinct subsets through an examination zone; and

(c) determining an identity and quantity of each specific subset of LSD target antigen of interest, if present, in the sample by (i) collecting data relating to one or more subsets of target capture microsphere classification parameters that distinguishes one target capture antibody microsphere of one subset from those of another target capture antibody microsphere subset according to a predetermined discriminate function table, including the fluorescence emission intensities, (ii) collecting data relating to the presence or absence of a corresponding subset of specific LSD antigen, (iii) quantifying each corresponding subset of specific LSD antigen on each subset of capture antibody microsphere.

33. The method of claim 32, further comprising: prior to step (b), adding a pooled population of detection antibodies to the exposed pooled population of the target capture microspheres, the pooled population of target detection antibodies having distinct subsets that correspond to and bind to the same specific subset of LSD antigens coupled to each distinct subset of the target capture microspheres, forming an exposed pooled population of target capture microsphere having distinct subsets.

34. The method of claim 32, wherein the detection antibodies include a label.

35. The method of claim 34, wherein each subset of target detection antibodies is conjugated to a fluorescent detection label.

36. The method of claim 32, wherein the sample is selected from a cellular extract, blood, plasma, or urine.

37. The method of claim 32, wherein the specific subset of LSD antigens is α-iduronidase, α-glucosidase, or saposin C.

38. The method of claim 32, wherein the specific subset of LSD antigens is β-glucosidase, α-galactosidase A, iduronate-2-sulphatase, α-iduronidase, N-acetylgalactosamine 4-sulphatase, galactose 6-sulphatase, acid sphingomyelinase, galactocerebrosidase, arylsulphatase A, saposin B, heparan-N-sulphatase, α-N-acetylglucosaminidase, acetylCoA: glucosamine N-acetyltransferase, N-acetylglucosamine 6-sulphatase, β-galactosidase, β-glucuronidase, aspartylglucosaminidase, acid lipase, β-hexosamindase A, β-hexosamindase B, GM2-acitvator, acid ceramidase, α-L-fucosidase, α-D-mannosidase, β-D-mannosidase, neuraminidase,

phosphotransferase, phosphotransferase g-subunit, palmitoyl protein thioesterase, tripeptidyl peptidase I, cathespsin K, α-galactosidase B, or sialic acid transporter.

39. The method of claim 32, wherein the specific subset of LSD antigens comprise an indicator of cell number, organelle number, cell size, organelle size, cell volume, or organelle volume.

40. The method of claim 39, wherein the specific subset of LSD antigens comprise LAMP-1, LAMP-2, saposin C, CD45 leukocyte common antigen, LIMP II, CD63.

41. The method of claim 32, wherein the microsphere has a diameter of about 5μm.

42. The method of claim 32, wherein the target capture microspheres in each distinct subset exhibit two or more characteristic fluorescence emission classification parameters.

43. The method of claim 32 wherein the target capture microspheres of one subset differ from the target capture microspheres of another subset in an intensity of at least one fluorescence emission classification parameter.

44. The method of claim 32, wherein the quantity of each specific subset of LSD target antigen of interest is proportional another specific subset of LSD target antigen of interest.

45. The method of claim 32, wherein results of said method are displayed in real time.

46. A method of screening for lysosomal storage disorder ("LSD") in a target biological

sample, the method comprising:

(a) exposing a pooled population of target capture microspheres to the target biological sample, the target capture microspheres having distinct subsets, and each distinct subset having: (i) one or more characteristic classification parameters that distinguishes one target capture microsphere of one subset from those of another target capture microsphere subset according to a predetermined discriminate microsphere function table; and (ii) a distinct capture antibody that can bind a specific subset of LSD antigens;

(b) adding a pooled population of detection antibodies to the exposed pooled population of the target capture microspheres, the pooled population of target detection antibodies having distinct subsets that correspond to and bind to the same specific subset of LSD antigens coupled to each distinct subset of the target capture microspheres, forming an exposed pooled population of target capture microsphere complexes having distinct subsets;

(c) passing the an exposed pooled population of target capture microsphere complexes having distinct subsets through an examination zone;

(d) determining the identity and quantity of each specific subset of LSD target antigen of interest, if present, in the sample by (i) collecting data relating to one or more subsets of target capture microsphere classification parameters that distinguishes one target capture antibody microsphere of one subset from those of another target capture antibody microsphere subset according to a predetermined discriminate function table, including the fluorescence emission intensities, (ii) collecting data relating to the presence or absence of a corresponding detection antibody that binds the subset of specific LSD antigen, (iii) quantifying each corresponding detection antibody on each subset of capture antibody microsphere; and

(e) comparing the identity and quantity of each specific subset of LSD target antigen of interest from the sample obtained from a patient having an unknown LSD clinical status to the identity and quantity of the same specific subset of LSD target antigen of interest from the sample obtained from a patient having a known LSD clinical status.

47. The method of claim 46, wherein the sample is selected from a cellular extract, blood, plasma, or urine.

48. The method of claim 46 wherein the specific subset of LSD antigens is α-iduronidase, α-glucosidase, or saposin C.

49. The method of claim 46, wherein the specific subset of LSD antigens is β-glucosidase, α-galactosidase A, Iduronate-2-sulphatase, α-iduronidase, N-acetylgalactosamine 4-sulphatase, galactose 6-sulphatase, acid sphingomyelinase, galactocerebrosidase, arylsulphatase A, saposin B, heparan-N-sulphatase, α-N-acetylglucosaminidase, acetylCoA: glucosamine N-acetyltransferase, N-acetylglucosamine 6-sulphatase, β-galactosidase, β-glucuronidase, aspartylglucosaminidase, acid lipase, β-hexosamindase A, β-hexosamindase B, GM2-acitvator, acid ceramidase, α-L-fucosidase, α-D-mannosidase, β-D-mannosidase, neuraminidase, phosphotransferase, phosphotransferase g-subunit, palmitoyl protein thioesterase, tripeptidyl peptidase I, cathespsin K, α-galactosidase B, or sialic acid transporter.

50. The method of claim 46, wherein the specific subset of LSD antigens comprise an indicator of cell number, organelle number, cell size, organelle size, cell volume, or organelle volume.

51. The method of claim 50, wherein the specific subset of LSD antigens comprise LAMP-1, LAMP-2, saposin C, CD45 leukocyte common antigen, LIMP II, CD63.

52. The method of claim 46, wherein the microsphere has a diameter of about 5μm.

53. The method of claim 46, wherein the target capture microspheres in each distinct subset exhibit two or more characteristic fluorescence emission classification parameters.

54. The method of claim 46 wherein the target capture microspheres of one subset differ from the target capture microspheres of another subset in an intensity of at least one fluorescence emission classification parameter.

55. The method of claim 46, wherein each subset of target detection antibodies is conjugated to a fluorescent detection label.

56. The method of claim 46, wherein the quantity of each specific subset of LSD target antigen of interest is proportional another specific subset of LSD target antigen of interest.

57. The method of claim 46, wherein results of said method are displayed in real time.

Dated this 8th day of August, 2003.

Women's and Children's Hospital
By its Patent Attorneys
MADDERNS

ABSTRACT

The present invention is generally related to a multiple screening diagnostic for Lysosomal Storage Disorders ("LSDs") and related diseases. More particularly, this invention pertains to compounds, reagents, and methods for identifying and quantifying multiple target enzymes and proteins that are used to accurately diagnose a LSD. These target enzymes and proteins are naturally present in biological fluids or tissues of patients. The invention also pertains to a Multiplexing Bead Technology for simultaneous screening of specific LSD enzymes.

Figure 1 of 6

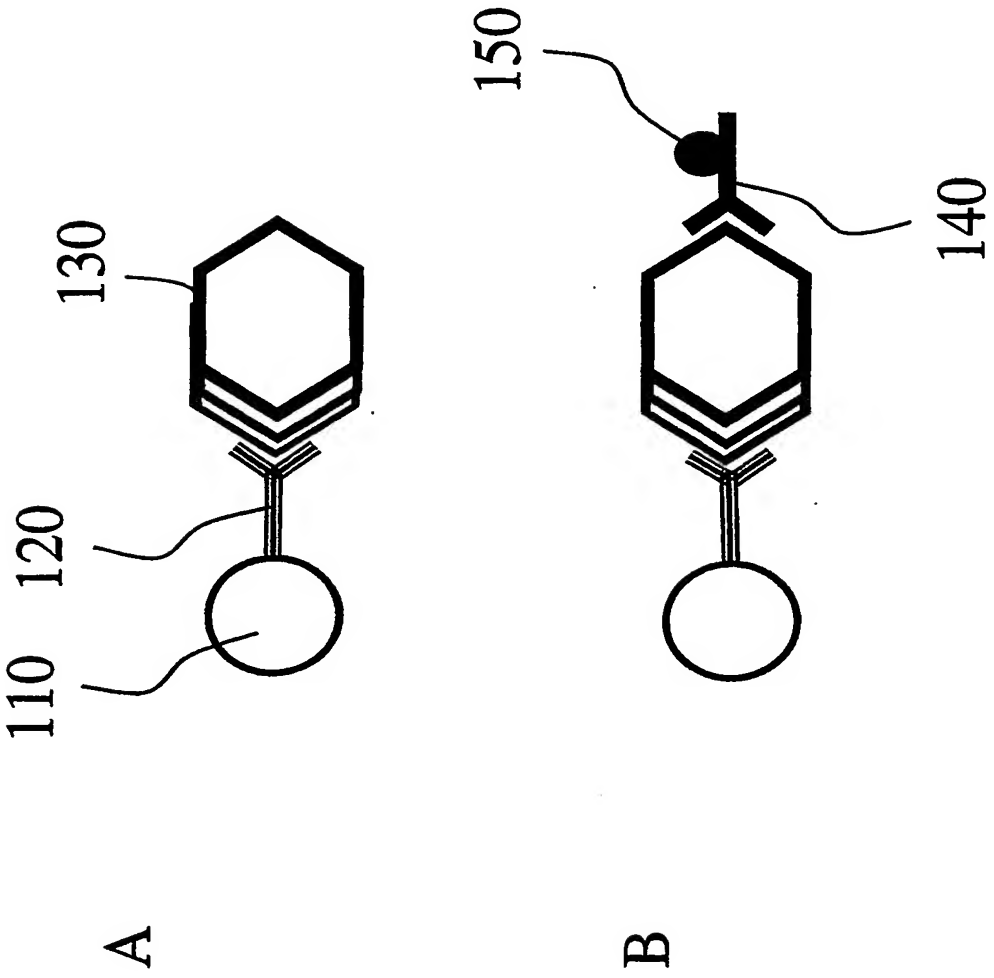


Figure 2 of 6

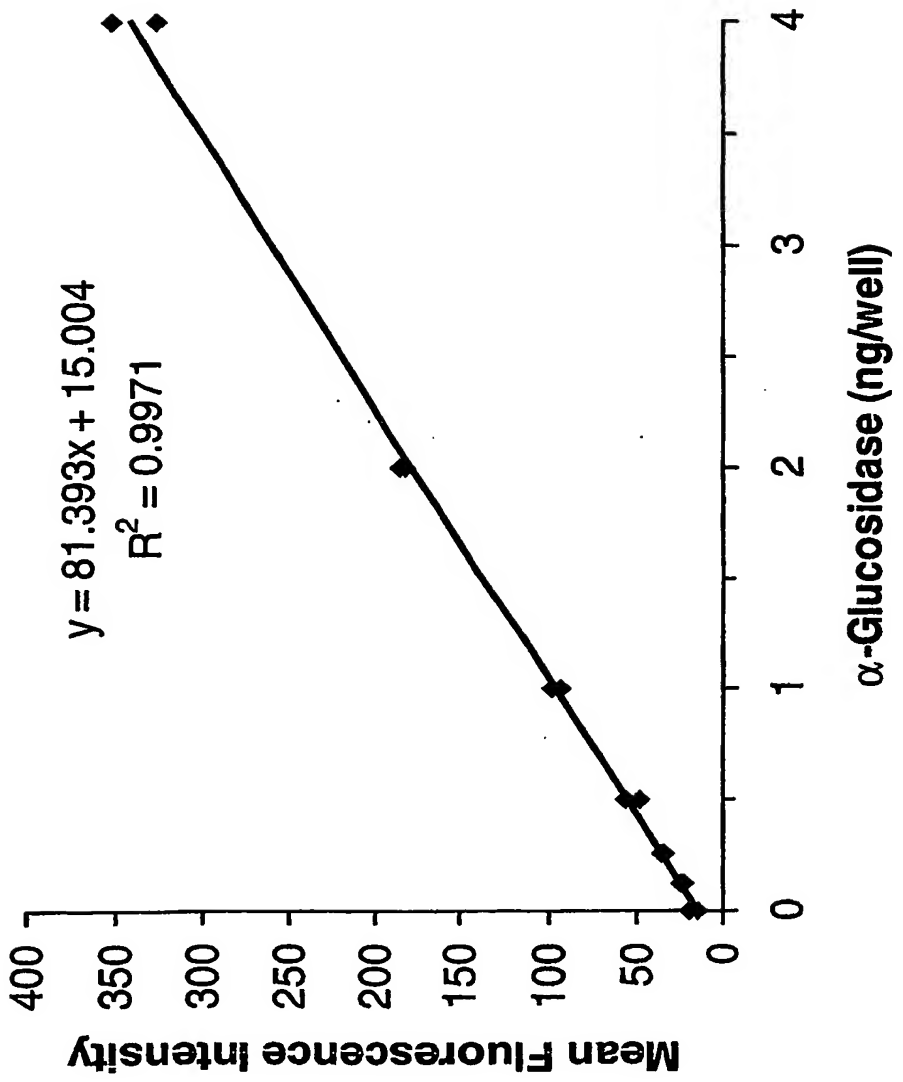
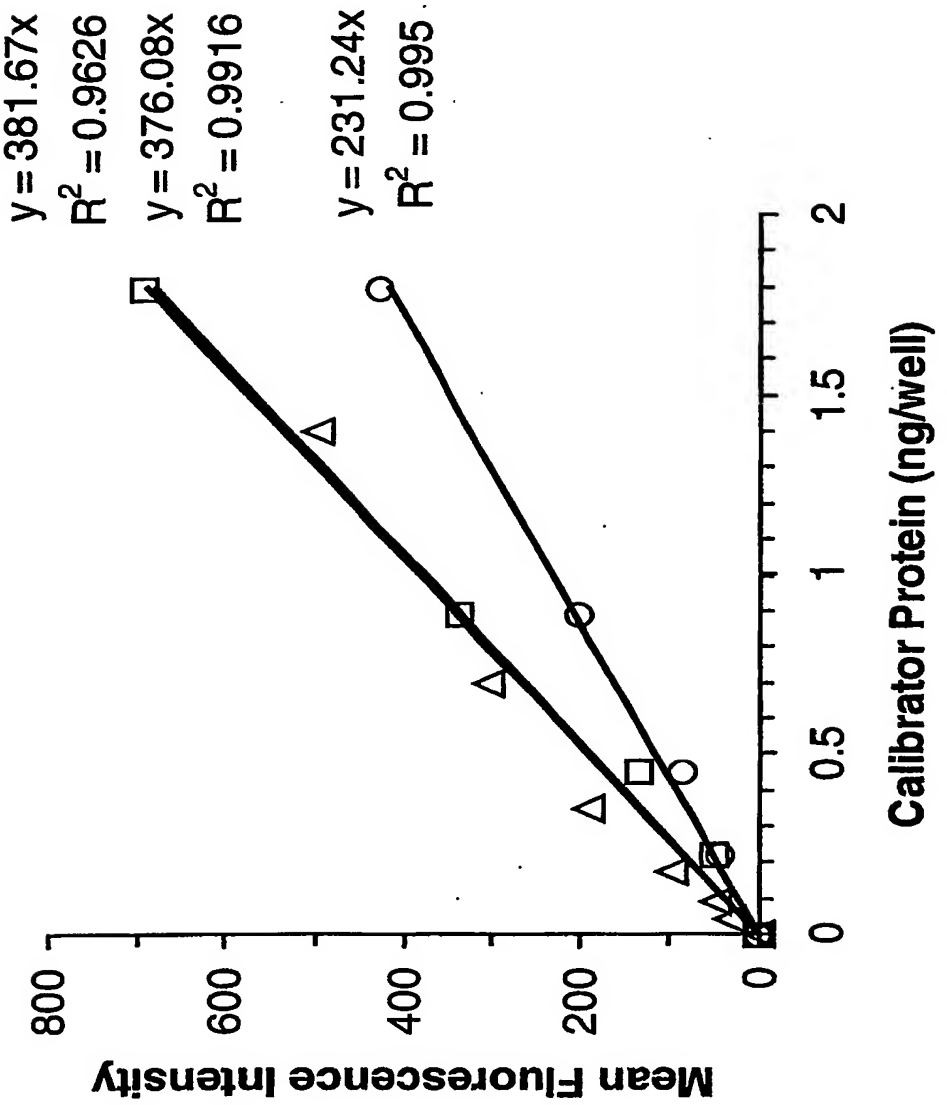

y = 81.393x + 15.004
R2 = 0.9971
Mean Fluorescence Intensity
0
50
100
150
200
250
300
350
400
0
1
2
3
4
α-Glucosidase (ng/well)

Figure 3 of 6


y = 381.67x
R2 = 0.9626
y = 376.08x
R2 = 0.9916
y = 231.24x
R2 = 0.995
Mean Fluorescence Intensity
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600
400
200
0
0
0.5
1
1.5
2
Calibrator Protein (ng/well)

Figure 4 of 6

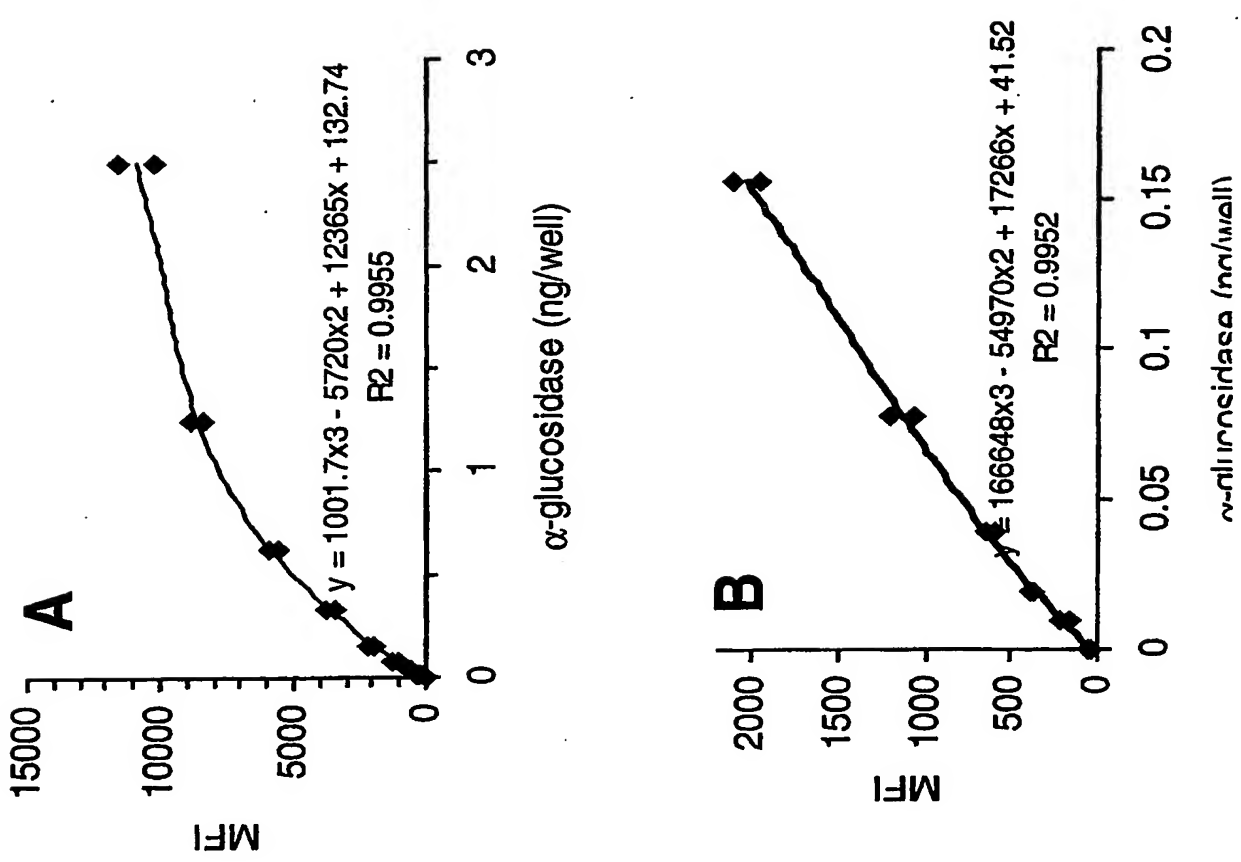
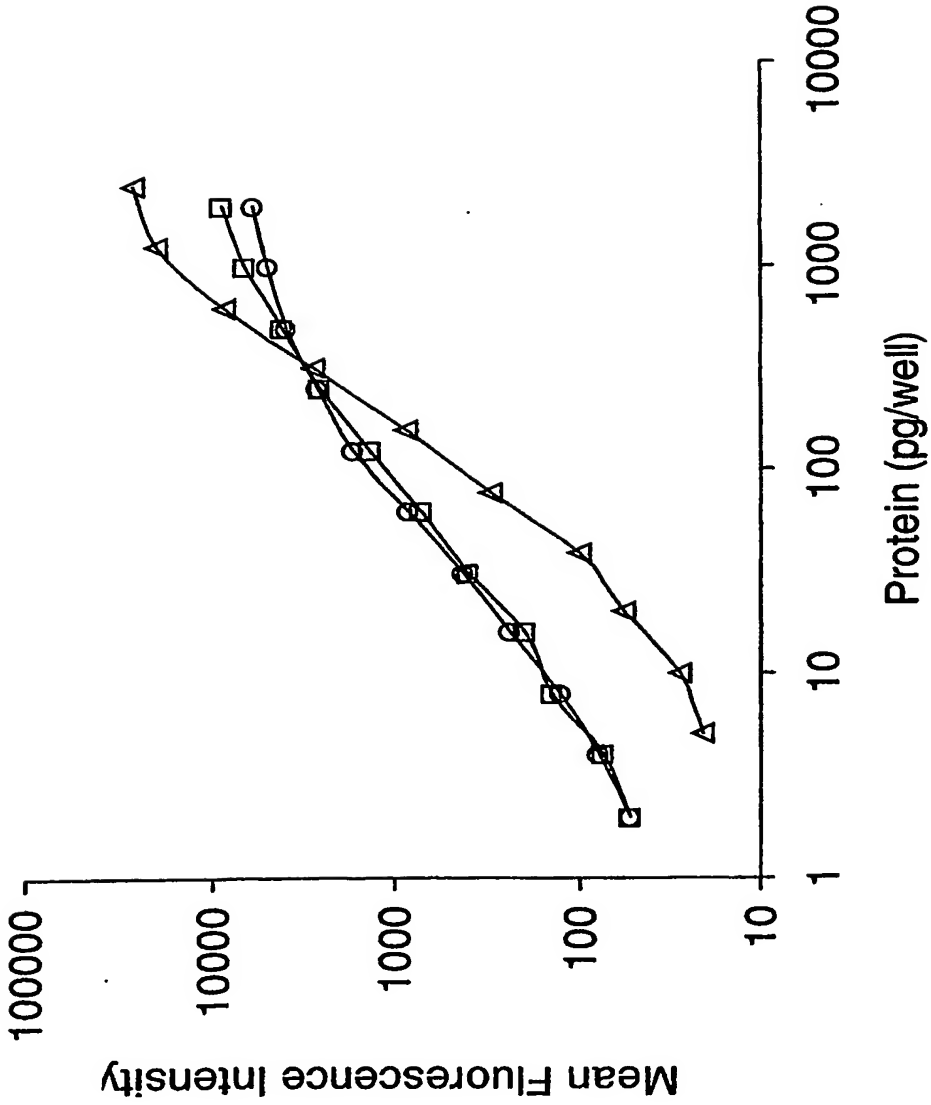

A
MFI
15000
10000
5000
0
y = 1001.7x3 - 5720x2 + 12365x + 132.74
R2 = 0.9955
0
1
2
3
α-glucosidase (ng/well)
B
MFI
2000
1500
1000
500
0
y = 166648x3 - 54970x2 + 17266x + 41.52
R2 = 0.9952
0
0.05
0.1
0.15
0.2

Figure 5 of 6


Mean Fluorescence Intensity
100000
10000
1000
100
10
1
10
100
1000
10000
Protein (pg/well)

Protein profiles/fingerprints for specific LSD

Analyte	Pompe	Gaucher	Fabry	MPS I	MPS II	MPS VI
LAMP-1	=	↑	=	↑	↑	↑
saposin C	↑	↑	↑	=	=	=
α-glucosidase,	↓	↑	=	↑	↑	↑
β-glucosidase,	=	↓	=	=	=	=
α-galactosidase,	=	↑	↓	=	=	=
α-iduronidase,	=	↑	=	↓	=	=
iduronate-2-sulphatase	=	↑	=	=	↓	=
N-acetylgalactosamine-4-sulphatase	=	↑	=	=	=	↓

↑ Indicates an increase relative to the control population
↓ Indicates a decrease relative to the control population
= Indicates no change relative to the control population

Figure 6 of 6